

Assessment of Dust Sampling Methods for the Study of Cultivable-Microorganism Exposure in Stables[∇]

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Received 17 June 2009/Accepted 5 October 2009

Studies have shown a link between living on a farm, exposure to microbial components (e.g., endotoxins or β -D-glucans), and a lower risk for allergic diseases and asthma. Due to the lack of validated sampling methods, studies of asthma and atopy have not relied on exposure assessment based on culture techniques. Our objective was therefore to compare several dust sampling methods for the detection of cultivable-microorganism exposure in stables. Sixteen French farms were sampled using four different methods: (i) active air sampling using a pump, (ii) passive dust sampling with a plastic box, (iii) dust sampling with an electrostatic dust fall collector (wipe), and (iv) dust sampling using a spatula to collect dust already settled on a windowsill. The results showed that collection of settled dust samples with either plastic boxes or wipes was reproducible (pairwise correlations, 0.72 and 0.73, respectively) and resulted in highly correlated results (pairwise correlation between the two methods, 0.82). We also found that settled dust samples collected with a plastic box correctly reflected the composition of the samples collected in the air of the stable when there was no farmer activity. A loss of microbial diversity was observed when dust was kept for 3 months at room temperature. We therefore conclude that measurement of viable microorganisms within a reasonable time frame gives an accurate representation of the microbial composition of stable air.

Several studies published in the early 2000s have shown that the risks of becoming atopic and presenting with symptoms of hay fever and asthma are lower for children who spend their early infancy on farms (2, 9, 13, 14, 26, 32). This protective effect was found to be associated with several factors, including contact with farm animals and high-level exposure to microbial components (including endotoxins, extracellular polysaccharides, and glucans) (33). The ALEX (ALlergy and EndotoXin) study showed that continual long-term exposure to stables until the age of 5 years was associated with the lowest frequencies of asthma, hay fever, and atopic sensitization (25). More recently, the PARSIFAL (Prevention of Allergy—Risk Factors for Sensitization Related to Farming and Anthroposophic Lifestyle) study confirmed the inverse association between bacterial endotoxins from mattress dust and atopic wheeze. This association was no longer significant, however, after adjustment for exposure to fungal beta(1,3)-glucans and fungal extracellular polysaccharides (28). In a recent review, von Mutius suggested that mold or other microbial components might modulate immune responses and thereby protect against allergic diseases, as previously suggested for endotoxins (33). Indeed, a study by Sudre et al. showed that huge amounts of pollen, bacteria, and fungi are found in the air of cowsheds (31). Last, this brief overview highlights the facts that (i) only a few antigens and

microbial substances have been studied and identified in the cowshed environment so far and (ii) it is likely, given the immunological mechanisms, that other microbes or compounds may be involved in the protective “farming effect.”

However, assessment of aerial microbial diversity in a large sample of farms and stables is difficult to perform. Ideally, such assessment would require the measurement of the inhaled dose of fungal spores and bacteria over a substantial period of time for each individual included in a cohort. Because such an approach is not applicable to large cohorts, settled-dust sampling has been proposed in numerous studies as an unsophisticated and cost-effective way to assess long-term exposure to airborne components (10, 11, 15, 17, 29, 30, 34). However, settled-dust analysis is only a surrogate measure for airborne exposure, and distortions between settled-dust and airborne-dust microbial flora should be evaluated. We present here a study aiming to assess whether the microbial composition of dust collected in cowsheds is representative of airborne microbial flora. In this study, we assess the richness and diversity of bacteria and fungi from airborne and settled dust collected by several methods in the cowsheds of 16 French farms during the winter season.

MATERIALS AND METHODS

Study design. The study was performed from February 2006 to April 2007 on 16 French farms located in the Franche-Comté region, an area located in the East of France between 46°35'4" to 47°21'9"N and 5°29'55" to 6°43'4"E. The altitude of the farms ranged from 200 m to 880 m. Ten of these farms corresponded to children who were previously included in the PASTURE study (35). Nine stables were freestall stables, and the other seven were stanchion stables. In the freestall stables, the cows were free to move until milking time; then they entered the milking room one after another. In the stanchion stables, cattle were

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[∇] Published ahead of print on 9 October 2009.

hobbled; cows were tied up all the time in the cowshed, and the farmer moved between them for milking.

Sample description and sampling. In each stable, one air sample was collected before the feeding session was started (before work), and the other was collected 20 min after the feeding session (after work). Collection of airborne dust was based on active impaction of air particles (with a 3-liter/min flow) using a cassette (diameter, 37 mm; Sensidyne, Clearwater, FL) containing a Teflon filter (pore diameter, 0.45 μ m; Millipore Sensidyne, Clearwater, FL) during a 20-min pumping session using a Gil-Air 3 pump (Sensidyne, Clearwater, FL). The device was fixed on a tripod at a fixed height of 75 cm and was located at a central place in the cowshed. The filters were rinsed with 10 ml of 0.1% Tween 80–sterile water (Sigma-Aldrich, Steinheim, Germany) and were shaken for 10 min in a stomacher (AES Laboratoire, Combourg, France) with a delay of less than 3 h after field work.

In addition, two settled-dust samples were collected using a spatula on either a windowsill or the metallic stanchion bars where the cows were hampered. In order to limit contamination by soil, samples were collected from supports located higher than 1.50 m and were put in a sterile plastic box.

Two other settled-dust samples were collected using plastic boxes (24 cm long, 18.6 cm wide, and 5.5 cm high; Mobil Plastic, France). An aluminum grid with a ca. 8-mm link was placed on top of the box to prevent large amalgams from settling inside. These boxes were left for 1 month in the cattle shed, out of reach of the cows. One set of dust samples (one spatula sample and one box sample) was analyzed on the day of sampling, and the other set was kept at room temperature for 3 months before being analyzed. Dust was transferred to a sterile plastic 40-ml tube by cleaning the box with a clean, thin brush under a hood. A total of 0.3 g of the dust (box-settled or spatula dust) was weighed and washed with 20 ml of Tween 80 at 0.1%. The mixture was thoroughly vortexed for 1 min. The solution obtained was used for culture and cold storage. This washing solution was used at different dilutions depending on the culture medium.

Culturing. A total of 250 μ l of the washing solution for the Teflon filter and 100 μ l of diluted dust were spread on petri dishes containing four different media: (i) dichloran-glycerol (Oxoid Ltd., Basingstoke, England) with 0.5% chloramphenicol (Sigma-Aldrich, Steinheim, Germany) at 30°C for mesophilic mold isolation, (ii) 3% malt agar (AES, Bruz, France) with 10% salt and 0.5% chloramphenicol for osmophilic fungal species, (iii) Difco actinomycete isolation agar (Becton Dickinson, le Pont de Claix, France) at 30°C for mesophilic actinomycetes, and (iv) R8 medium prepared according to the method of Amner and colleagues at 52°C for the detection of thermophilic actinomycetes (1). After 7 days of incubation, colonies of molds and actinomycetes were identified using macroscopic and microscopic criteria. Another 250 μ l of washing solution and 100 μ l of diluted dust were cultured on Mueller-Hinton (MH) agar (Becton Dickinson). Bacterial colonies were counted after a 48-h incubation and were reisolated by subculturing on MH agar according to the colony morphology and the Gram staining result.

DNA extraction and PCR amplification. Bacterial DNA was further extracted as described by Drancourt et al. (6), and 100 μ l of the supernatant was collected. fD1 and rD1 were used as primers for 16S rRNA gene PCR (36). DNA was amplified with 0.5 μ M primers fD1 and rD1, 200 μ M deoxynucleoside triphosphates, 1 mM MgCl₂, 1 \times REDTaq buffer, 0.01 U of REDTaq, and 1 μ l of the DNA sample. The reaction mixture was subjected to the PCR program as reported by Paster et al. (21). The amplification products of each reaction were analyzed by electrophoresis for 60 min at 80 V in agarose (1.5%) gels. DNA bands were visualized by UV transillumination. For sequencing, the mixture contained DNA polymerase, pyrophosphatase, buffer, deoxynucleoside triphosphates, dye terminators, DNA templates, water, and the internal reverse primer P-519 (5'-GTA TTA CCG CGG CTG CTG GCA C-3') to sequence the 497-bp fragment. The sequencing mixture was subjected to 35 cycles consisting of 20 s of denaturation at 96°C, 20 s of primer annealing at 50°C, and 2 min of primer extension at 60°C. The reading was performed with a Ceq 8000 genetic analysis system (Beckman Coulter), and results were compared to the NCBI GenBank database (<http://www.ncbi.nlm.nih.gov>) using BLAST (<http://www.ncbi.nlm.nih.gov/blast>). Molecular identification at the genus level was defined by a sequence similarity higher than 97% (12, 22). Results are expressed in CFU per cubic meter or per gram of dust, depending on the sample analyzed.

Second winter sampling. Four additional settled-dust samples were collected on 13 of the farms during a second winter using plastic boxes (two repeated samples: W₂Box₁ and W₂Box₂) and wipes (two repeated samples: W₂Wipe₁ and W₂Wipe₂) in order to assess the reproducibility of the results. Box-settled dust from the second winter was handled the same way as dust from the first winter. Sterile wipes (10 by 12 cm) were placed down flat in a plastic lab tip box and were left for 15 days in the cowshed next to the box-settled dust collector. The wipes were then put in a sterile bag with 20 ml of Tween 80 at 0.1% and were shaken

for 10 min in a stomacher. A total of 100 μ l of the washing solution was added to petri dishes with different dilutions spanning the ranges of microorganisms (from pure washing solution to the solution diluted 10³-fold), and the microorganisms were treated as described above. The sampling strategy is depicted in Fig. 1.

A total of 92 samples were analyzed for the first winter, and 52 samples were analyzed for the second winter.

Statistical analyses. Comparisons of air measurements before and after the feeding session and between storage periods (no storage versus 3 months of storage) were conducted using the Wilcoxon matched-pair signed-rank test for each species or genus. The pairwise correlation coefficients between sampling techniques were computed. To account for multiple testing, *P* values were adjusted by the Bonferroni procedure. Statistical analyses were performed using STATA software (version 8.2; StataCorp LP, College Station, TX).

Species accumulation curves were constructed using EstimateS software (version 8.0; R. K. Colwell) to estimate the diversity of the samples. Mao Tau indices obtained with this software were then plotted.

RESULTS

Genus and species identification. Twenty fungal taxa were identified in the different samples via the culture methods. The most frequent fungal genera identified in the dusts were *Eurotium* (mainly *Eurotium amstelodami* and *Eurotium umbrosum* [46.6% of the total amount of fungi in the air and 34.8% of the total in the dust]), *Wallemia* (25.1% and 19.8%), *Aspergillus* (10.5% and 10.4%), and *Penicillium* (7.0% and 10.4%). The other fungi identified belonged to the genera *Absidia*, *Mucor*, *Rhizopus*, *Syncephalastrum*, *Fusarium*, *Alternaria*, *Aureobasidium*, *Acremonium*, *Scopulariopsis*, *Cladosporium*, *Humicola*, and *Trichosporon*. Some fungi could not be identified at the genus level. They were classified as unidentified moniliaceae, unidentified dematiaceae, red yeasts (mainly *Rhodotorula*), and white yeasts (*Candida* spp., *Cryptococcus* spp., and *Debaryomyces* spp.). Bacterial colonies collected from MH medium were classified according to Gram staining, which allowed us to identify 57.4% of gram-positive cocci in the air and 68.2% in the dust, 32.2% of gram-positive bacilli in the air and 31.6% in the dust, and 10.3% of gram-negative bacilli in the air and 29.4% in the dust. No gram-negative cocci were identified. Three species of actinobacteria cultured on Difco actinomycete isolation agar and R8 medium were identified at the species level (*Saccharomonospora viridis*, *Saccharopolyspora rectivirgula*, and *Thermoactinomyces vulgaris*), whereas the other actinobacteria were grouped depending on their growth temperatures (mesophilic *Actinomycetales* and thermophilic *Actinomycetales*). Overall, partial 16S rRNA gene sequencing of bacterial colonies collected from MH medium led to the identification of 93 bacterial taxa corresponding to 41 bacterial genera. The main bacterial genera identified by biomolecular methods were *Staphylococcus* (mainly *Staphylococcus equorum* and *Staphylococcus xylosus* [24.2% of the total amount of culturable bacteria in the air and 39.2% of that in the dust]), *Curtobacterium* (2.3% and 3.8%), *Bacillus* (3.4% and 2.9%), and *Brachybacterium* (0.5% and 11.4%). The detailed results of this identification will be reported in a further paper concerning an inventory of the microbial farming environment. To simplify the presentation of results, we merged the microorganisms into the following 12 main groups: (i) fungi belonging to the order *Mucorales* (total *Mucorales*), (ii) *Cladosporium* species, (iii) *Wallemia sebi*, (iv) fungi from the *Eurotium* and *Aspergillus* genera, (v) other fungal species, (vi) yeasts (white and red), (vii) gram-negative bacilli, (viii) gram-positive bacilli,

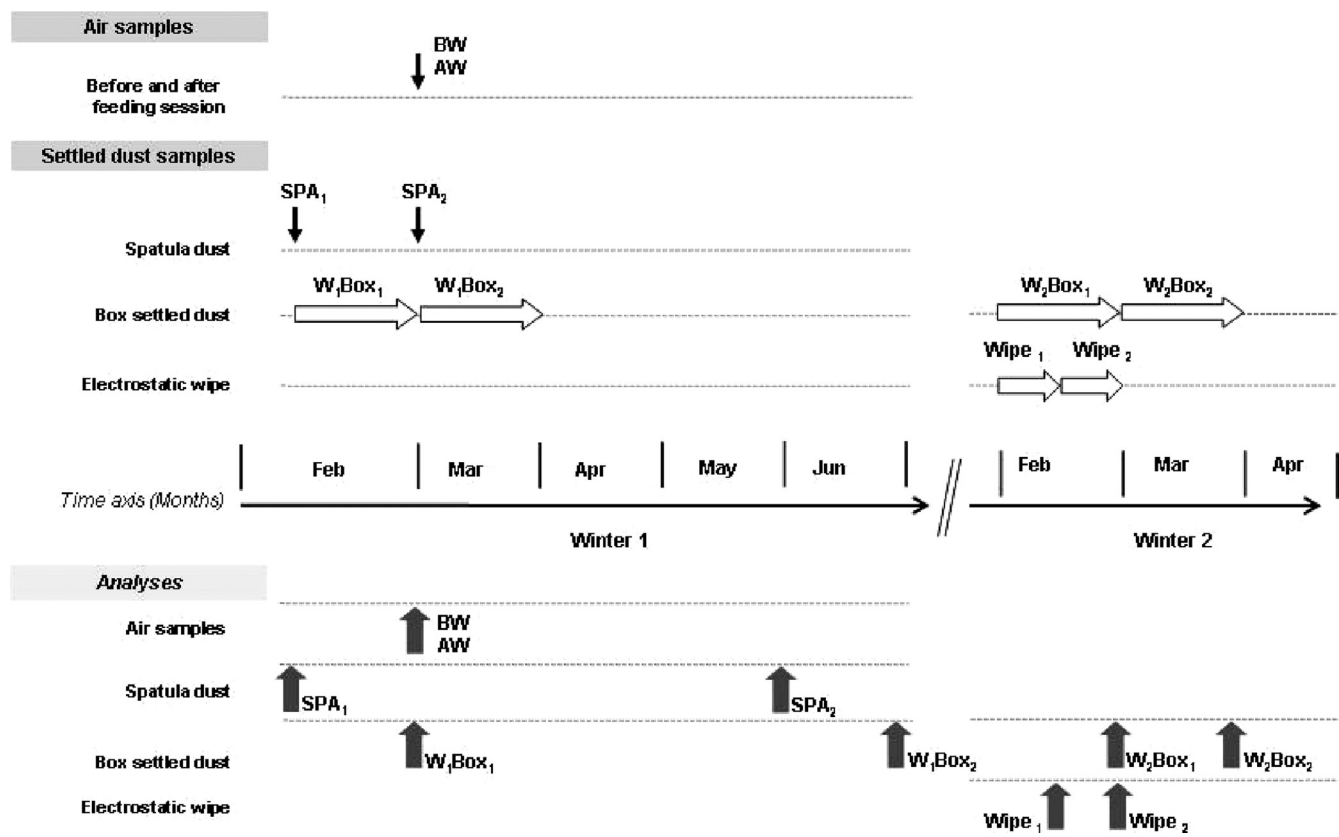


FIG. 1. Study design. Horizontal sampling arrows represent sampling over a long period (here, 15 days or 1 month). Vertical sampling arrows correspond to a short sampling time (20 min of air sampling or spatula dust sampling). The time axis is graduated into months for the first (2006) and second (2007) winters. W₁Box₁ and W₁Box₂, box-settled dust samples 1 and 2 from the first winter; W₁SPA₁ and W₁SPA₂, spatula-settled dust samples 1 and 2 from the first winter; BW, air before cattle feeding; AW, air after cattle feeding; W₂Box₁ and W₂Box₂, box-settled dust samples 1 and 2 from the second winter; W₂Wipe₁ and W₂Wipe₂, wipe-settled dust samples 1 and 2 from the second winter.

(ix) gram-negative cocci, (x) gram-positive cocci, (xi) mesophilic actinobacteria, and (xii) thermophilic actinobacteria.

Airborne and settled-dust microflora. The microbiological compositions of the airborne flora (before and after cattle feeding) and of the settled dust collected during the first winter are shown in Fig. 2. Before feeding, the airborne microflora of the stables was dominated by *Aspergillus/Eurotium* fungi and gram-positive cocci; these two groups were surpassed by a significant amount of mesophilic actinomycetes after handling of the hay. The total number of bacteria and fungi after the feeding session was higher than that before the feeding session. This increase was significant for *Eurotium* spp. ($P = 0.004$) and *Cladosporium* spp. ($P = 0.004$) (Wilcoxon matched-pair signed-rank test). Figure 2 shows that the settled dust collected in plastic boxes indicated a similar microbial composition of air before the feeding of the cattle, with a predominance of *Aspergillus/Eurotium* and gram-positive bacteria. Settled dust collected using a spatula seemed less representative of the airborne microflora, due to the relative overrepresentation of the gram-positive cocci. Pairwise correlation tests with regard to the counting of the 12 main groups showed only a weak relationship between the airborne and settled-dust samples (Table 1). However, the correlation between the airborne and settled-dust microfloras was much better, especially when the mean values for the 16 farms rather than the individual values

for each farm were considered (Table 1). The best results were obtained for comparisons of the air before the feeding session and settled dust collected in a plastic box (correlation coefficient, 0.82).

The fungal species accumulation curves performed from the two air samples and the two settled-dust samples showed that 16 farms were sufficient for correct assessment of the fungal diversity within farms (Fig. 3). These data also showed that a plateau was reached more rapidly when settled dust (collected with a plastic box or a spatula) was analyzed than when airborne dust was analyzed. In contrast, the slope of the curve did not reach any plateau for bacterial results. Regardless of the type of samples (airborne or settled dust) analyzed, therefore, these results show that a set of 16 samples is not sufficient to assess the bacterial diversity in these stables (Fig. 3).

Overall, a 3-month storage period at room temperature lowered microbial diversity in the dust samples significantly, as shown for the plastic boxes (Fig. 4) (Wilcoxon tests). In particular, we noted decreases in the levels of gram-positive cocci ($P = 0.005$), *Cladosporium cladosporioides* ($P = 0.009$), and white yeast groups ($P = 0.04$) and relative increases in those of *Aspergillus versicolor* ($P = 0.004$), mesophilic *Actinomycetes* ($P = 0.02$), *Absidia cylindrospora* ($P = 0.03$), and *Scopulariopsis brevicaulis* ($P = 0.04$). The same comparison was performed for spatula-settled dust. This comparison

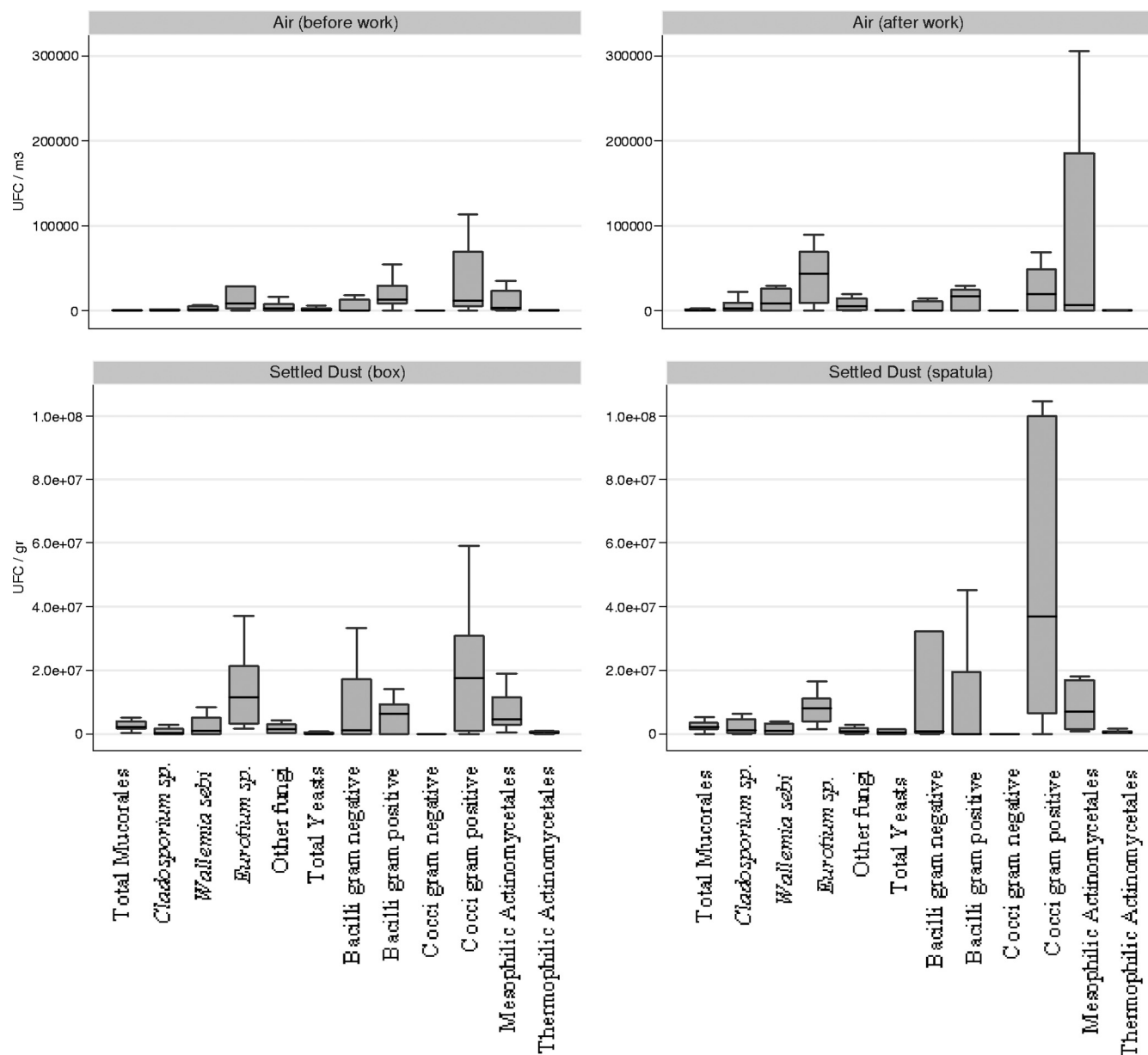


FIG. 2. Box plot profiles of the four sampling types for the first winter: air before and after work (cattle feeding), dust that had settled on a plastic box over 1 month, and dust that had settled on a dry surface and was harvested with a spatula. The bar in the middle of each box represents the median, and the lower and upper hinges represent the 25th and 75th percentiles, respectively. The whiskers correspond to the adjacent values of the lower and upper hinges. For the graphical representation, outside values were not plotted in order to produce an easily readable presentation of the data.

showed decreases in the levels of *Cladosporium cladosporioides* ($P = 0.005$), white yeasts ($P = 0.009$), red yeasts ($P = 0.01$), gram-negative bacilli ($P = 0.02$), *Eurotium* spp. ($P = 0.04$), and thermophilic *Actinomycetes* ($P = 0.04$) and an increase in the level of *Scopulariopsis brevicaulis* ($P = 0.04$).

Reproducibility of microbial recovery. The pairwise correlation test performed with the dust samples collected on 13 farms during the second winter showed that both the plastic box and wipe sampling methods exhibited good reproducibility (Table 2). This test also revealed a strong correlation between the results obtained with the plastic boxes and the wipes (Table 2).

In particular, the results corresponding to the two wipes left for two consecutive periods of 15 days were strongly correlated with those of the plastic box left for 1 month during exactly the same period. Indeed, a pairwise correlation of 0.82 means that these two types of samples shared 67% of their variability.

DISCUSSION

The composition of airborne or settled dust is complex and is known to be subject to daily and seasonal changes (20). Some studies highlight the relationships between exposure to

TABLE 1. Pairwise correlation coefficients for the different types of samples from the first winter

Sample type	Correlation coefficients (ind, mean) between samples ^a			
	Air		Dust	
	Before work	After work	W ₁ Box ₁	W ₁ Spa ₁
Air				
Before work	1			
After work	0.33**, 0.76*	1		
Dust				
W ₁ Box ₁	0.09**, 0.82*	0.16**, 0.35*	1	
W ₁ Spa ₁	0.18**, 0.69*	0.18**, 0.47*	0.03**, .59*	1

^a Correlation coefficients between air and dust samples for the first winter were calculated according to two methods. In the first method, each individual value for every microbial taxon was taken into account (ind) (**, $P < 0.01$). In the second method, the mean value for every microbial taxon within the 16 farms was calculated (mean) (*, $P < 0.05$).

microbial components (e.g., endotoxins, ergosterols, or β -D-glucans) and several health outcomes, including a lower risk for atopy and asthma among children raised on farms (3, 5, 7). However, it is not clear whether these molecules are responsible for the protective effect of farm environments against asthma and atopy. Therefore, there is a need to better investigate microbial biodiversity in farm environments and, in particular, to identify sampling methods that may help to assess the microbial composition of airborne dust. To the best of our knowledge, this study is the first aiming to assess the sampling methods with regard to microbial diversity in cowsheds. Even though other methods to identify microorganisms in the environment without culture steps (such as cloning [19] or denaturing gradient gel electrophoresis [16]) have already been reported, we chose not to use them, because they are time-

consuming, they are expensive given the number of samples, and they show only part of the microbial picture. Denaturing gradient gel electrophoresis or similar methods might eventually be more powerful analytically, but they have the disadvantage that they do not assess viability. In addition, our main objective was to assess whether the microbial composition of dust collected in cowsheds was representative of airborne microbial flora, not to provide an exhaustive overview of the microbial diversity in the air and settled dust.

Overall, the study showed that settled-dust sample collection with either plastic boxes or wipes was reproducible and resulted in highly correlated results (pairwise correlation, 0.82). The results also showed that settled-dust samples collected with a plastic box correctly reflected the composition of the air samples harvested in the stable before cattle feeding (pairwise correlation, 0.82 for the mean values obtained from the 16 farms). This complements the previous work of Noss et al., who showed that the use of electrostatic wipes was a valid method for measuring the average airborne endotoxin exposure in indoor environments (18). For this exposure assessment, we focused on culturable bacteria and fungi that were easily obtainable using standard culture media. We acknowledge, however, that these microorganisms represent only a subfraction of the microorganisms in the air of the cowsheds (8), because numerous bacteria and fungi are known to be unculturable or to require various specific conditions for culture. We also decided to focus on the winter, because during this season the numbers of microorganisms are high in stables due to the continuous presence of the cattle and to feeding in an enclosed environment (23, 27). Despite these limitations, our results highlight key points that need to be taken into account in order to ensure better reliability of settled-dust sampling. First, the choice of an appropriate sampling device is impor-

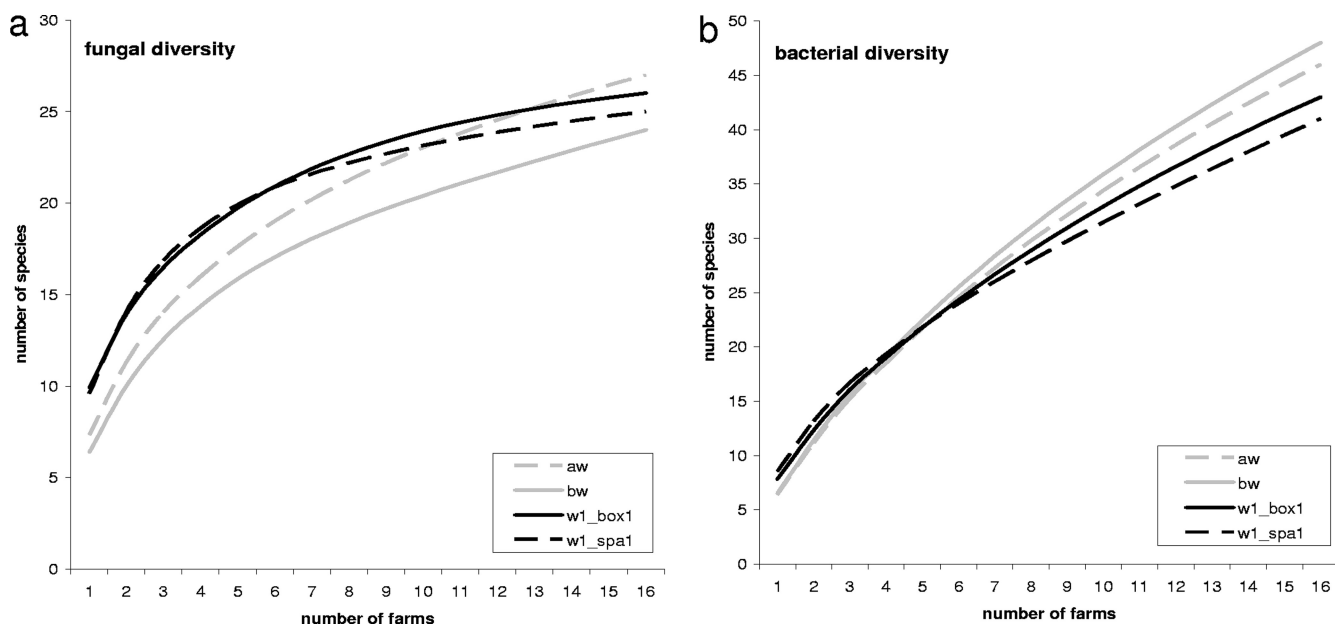


FIG. 3. Species accumulation curves for airborne dust and settled dust samples during the first winter. (a) Fungal species accumulation curves; (b) bacterial species accumulation curves. Gray lines represent the air (bw, before cattle feeding [solid line]; aw, after cattle feeding [dashed line]). Black lines represent the settled dust analyzed on the day of the sampling (w₁_box₁, box-settled dust [solid line]; w₁_spa₁, spatula-settled dust [dashed line]).

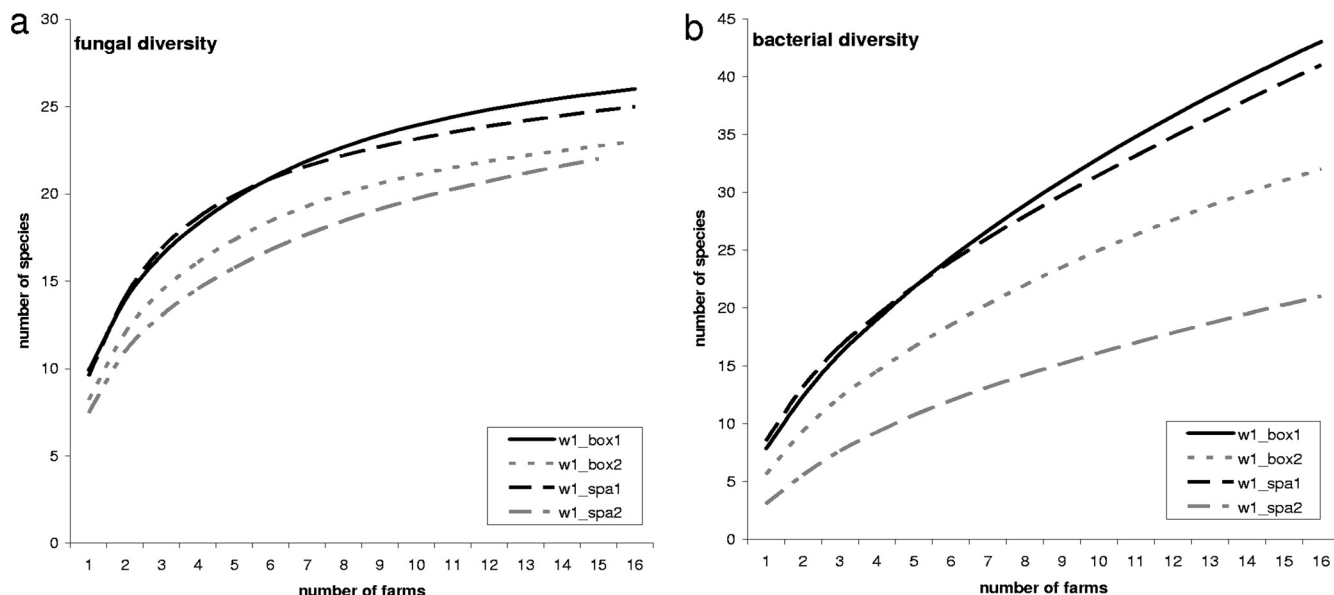


FIG. 4. Species accumulation curves for settled dust samples during the first winter. The effect of 3 months of storage at room temperature on microbial diversity are shown. (a) Fungal species accumulation curves; (b) bacterial species accumulation curves. Black lines represent the dust samples (w1_box1, box-settled dust sample 1; w1_spa1, spatula-settled dust sample 1) analyzed on the day of the sampling. Gray lines represent the samples analyzed after 3 months at room temperature (w1_box2, box-settled dust sample 2; w1_spa2, spatula-settled dust sample 2).

tant, as shown by the differences observed between the results obtained with a plastic box versus a spatula. Second, microbial analysis can be postponed to a limited extent in large studies for logistical reasons. If these two points are not considered, the evolution of the microflora in the dust results in the disappearance of some species (with a loss of diversity after a 3-month storage period) and the predominance of other species (such as gram-positive bacteria in settled dust collected with spatulas). The evolution of the microflora in settled dust with the passage of time may partially explain the poor relationships between the air and settled-dust results observed in previous studies. Chew et al. studied the results of fungal cultures derived from floor dust and indoor air samples in 397 American homes (4), demonstrating that the discrepancy between settled-dust and airborne-dust analyses may also reflect

the poor representativeness of air samples collected during a unique and short period. Our results also corroborated this conclusion, since we reported a lower correlation between settled dust and individual air sampling results than the correlation obtained when mean values were considered. This poor correlation between air sampling and settled-dust methods obtained when each farm was considered separately may therefore be indicative of the high variability of the microbial composition of airborne samples over time, even during the same day (20, 31). For instance, hay distribution resulted in increases in the levels of *Eurotium* and *Cladosporium*, molds known to use hay as a substrate for growth. Similarly, we found that mesophilic actinomycetes, which are bacteria usually found in the hay, were dominant in the airborne dust after hay handling but not before. These variations in the airborne composition of the air within stables, in relation to the substrates distributed, have been observed by several authors (24, 27). To improve the correlation between air and dust methods, a longer air sampling period in the stable may be considered, but active sampling would induce a loss in the microbial diversity by saturating the filter and desiccating the microorganisms. In contrast to air sampling, the settled-dust collection methods allow cumulative sampling over several weeks; this prolonged period might be an advantage for exposure assessment. It is noteworthy that microbial diversity was greater in the settled-dust samples than in the airborne dust, even for samples collected just after handling of the hay. It is likely that some of the microorganisms found in the settled dust might arise from previous manipulation of substrates containing species that were not present in the substrates manipulated on the day of the air sampling.

In conclusion, because of their standardization and reproducibility, settled-dust sampling methods performed using a box or an electrostatic wipe appear to be reliable ways of

TABLE 2. Pairwise correlation coefficients for the samples from the second winter

Dust collection method	Correlation coefficient ^a				
	Wipe			Box	
	W ₂ Wipe ₁	W ₂ Wipe ₂	W ₂ Wipe	W ₂ Box ₁	W ₂ Box ₂
Wipes					
W ₂ Wipe ₁	1				
W ₂ Wipe ₂	0.73*	1			
Boxes					
W ₂ Box ₁	0.78*	0.77*	0.82*	1	
W ₂ Box ₂	0.63*	0.57*	0.64*	0.72*	1

^a Every individual value for every microbial taxon was taken into account in the calculation of the correlation coefficients between the dust collection methods of the second winter season. W₂Wipe₁ and W₂Wipe₂ were placed successively in the same spot of the barn, as were W₂Box₁ and W₂Box₂. The W₂Wipe column corresponds to the compilation of W₂Wipe₁ and W₂Wipe₂ results, and it also corresponds to the same time period as W₂Box₁. *, $P < 0.01$.

assessing the composition of the airborne dust in cowsheds and to be applicable in large field studies of health effects. Because these samples can also be deployed in large-scale population studies, this work opens the field for studies investigating the relationships between airborne-microorganism diversity in a stable and protection against allergy and asthma in this specific environment.

ACKNOWLEDGMENTS

The work was financially supported by the Chambre Régionale d'Agriculture de Franche-Comté and by the Conseil Régional de Franche-Comté. This work was also partly supported by the European Union through the GABRIEL project for testing of the wipe method on cultivable microorganisms.

We thank Thierry Dubief and Michel Billot for technical assistance and the French farmers who participated in the project.

REFERENCES

- Amner, W., C. Edwards, and A. J. McCarthy. 1989. Improved medium for recovery and enumeration of the farmer's lung organism, *Saccharomonospora viridis*. *Appl. Environ. Microbiol.* **55**:2669–2674.
- Braun-Fahrlander, C., M. Gassner, L. Grize, U. Neu, F. H. Sennhauser, H. S. Varonier, J. C. Vuille, B. Wuthrich, et al. 1999. Prevalence of hay fever and allergic sensitization in farmer's children and their peers living in the same rural community. *Clin. Exp. Allergy* **29**:28–34.
- Braun-Fahrlander, C., J. Riedler, U. Herz, W. Eder, M. Waser, L. Grize, S. Maisch, D. Carr, F. Gerlach, A. Bufe, R. P. Lauener, R. Schierl, H. Renz, D. Nowak, and E. von Mutius. 2002. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N. Engl. J. Med.* **347**:869–877.
- Chew, G. L., C. Rogers, H. A. Burge, M. L. Muilenberg, and D. R. Gold. 2003. Dustborne and airborne fungal propagules represent a different spectrum of fungi with differing relations to home characteristics. *Allergy* **58**:13–20.
- Douwes, J., G. Le Gros, P. Gibson, and N. Pearce. 2004. Can bacterial endotoxin exposure reverse atopy and atopic disease? *J. Allergy Clin. Immunol.* **114**:1051–1054.
- Drancourt, M., C. Bollet, A. Carlioz, R. Martelin, J. P. Gayral, and D. Raoult. 2000. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *J. Clin. Microbiol.* **38**:3623–3630.
- Eder, W., W. Klimecki, L. Yu, E. von Mutius, J. Riedler, C. Braun-Fahrlander, D. Nowak, and F. D. Martinez. 2004. Toll-like receptor 2 as a major gene for asthma in children of European farmers. *J. Allergy Clin. Immunol.* **113**:482–488.
- Eduard, W., and D. Heederik. 1998. Methods for quantitative assessment of airborne levels of noninfectious microorganisms in highly contaminated work environments. *Am. Ind. Hyg. Assoc. J.* **59**:113–127.
- Ernst, P., and Y. Cormier. 2000. Relative scarcity of asthma and atopy among rural adolescents raised on a farm. *Am. J. Respir. Crit. Care Med.* **161**:1563–1566.
- Halstensen, A. S., K. C. Nordby, O. Elen, and W. Eduard. 2004. Ochratoxin A in grain dust—estimated exposure and relations to agricultural practices in grain production. *Ann. Agric. Environ. Med.* **11**:245–254.
- Heederik, D., P. S. Thorne, and G. Doekes. 2002. Health-based occupational exposure limits for high molecular weight sensitizers: how long is the road we must travel? *Ann. Occup. Hyg.* **46**:439–446.
- Janda, J. M., and S. L. Abbott. 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J. Clin. Microbiol.* **45**:2761–2764.
- Kilpeläinen, M., E. O. Terho, H. Helenius, and M. Koskenvuo. 2000. Farm environment in childhood prevents the development of allergies. *Clin. Exp. Allergy* **30**:201–208.
- Klintberg, B., N. Berglund, G. Lilja, M. Wickman, and M. van Hage-Hamsten. 2001. Fewer allergic respiratory disorders among farmers' children in a closed birth cohort from Sweden. *Eur. Respir. J.* **17**:1151–1157.
- Krysińska-Traczyk, E., J. Perkowski, and J. Dutkiewicz. 2007. Levels of fungi and mycotoxins in the samples of grain and grain dust collected from five various cereal crops in eastern Poland. *Ann. Agric. Environ. Med.* **14**:159–167.
- Liu, X. C., Y. Zhang, M. Yang, Z. Y. Wang, and W. Z. Lv. 2007. Analysis of bacterial community structures in two sewage treatment plants with different sludge properties and treatment performance by nested PCR-DGGE method. *J. Environ. Sci. (China)* **19**:60–66.
- Moloczniak, A. 2002. Qualitative and quantitative analysis of agricultural dust in working environment. *Ann. Agric. Environ. Med.* **9**:71–78.
- Noss, I., I. M. Wouters, M. Visser, D. J. Heederik, P. S. Thorne, B. Brunekreef, and G. Doekes. 2008. Evaluation of a low-cost electrostatic dust fall collector for indoor air endotoxin exposure assessment. *Appl. Environ. Microbiol.* **74**:5621–5627.
- Pakarinen, J., A. Hyvarinen, M. Salkinoja-Salonen, S. Laitinen, A. Nevalainen, M. J. Makela, T. Haahntela, and L. von Hertzen. 2008. Predominance of Gram-positive bacteria in house dust in the low-allergy risk Russian Karelia. *Environ. Microbiol.* **10**:3317–3325.
- Pangloli, P., Y. Dje, O. Ahmed, C. A. Doane, S. P. Oliver, and F. A. Draughon. 2008. Seasonal incidence and molecular characterization of *Salmonella* from dairy cows, calves, and farm environment. *Foodborne Pathog. Dis.* **5**:87–96.
- Paster, B. J., M. K. Russell, T. Alpagot, A. M. Lee, S. K. Boches, J. L. Galvin, and F. E. Dewhirst. 2002. Bacterial diversity in necrotizing ulcerative periodontitis in HIV-positive subjects. *Ann. Periodontol.* **7**:8–16.
- Patel, J. B. 2001. 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. *Mol. Diagn.* **6**:313–321.
- Reboux, G., R. Piarroux, F. Mauny, A. Madroszyk, L. Millon, K. Bardonnet, and J. C. Dalphin. 2001. Role of molds in farmer's lung disease in eastern France. *Am. J. Respir. Crit. Care Med.* **163**:1534–1539.
- Reboux, G., M. Reiman, S. Rousset, K. Taattola, L. Millon, J. C. Dalphin, and R. Piarroux. 2006. Impact of agricultural practices on microbiology of hay, silage and flour on Finnish and French farms. *Ann. Agric. Environ. Med.* **13**:267–273.
- Riedler, J., C. Braun-Fahrlander, W. Eder, M. Schreuer, M. Waser, S. Maisch, D. Carr, R. Schierl, D. Nowak, and E. von Mutius. 2001. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. *Lancet* **358**:1129–1133.
- Riedler, J., W. Eder, G. Oberfeld, and M. Schreuer. 2000. Austrian children living on a farm have less hay fever, asthma and allergic sensitization. *Clin. Exp. Allergy* **30**:194–200.
- Rousset, S., G. Reboux, J. C. Dalphin, K. Bardonnet, L. Millon, and R. Piarroux. 2004. Microbiological evolution of hay and relapse in patients with farmer's lung. *Occup. Environ. Med.* **61**:e3.
- Schram-Bijkerk, D., G. Doekes, J. Douwes, M. Bovee, J. Riedler, E. Ublagger, E. von Mutius, M. R. Benz, G. Pershagen, M. van Hage, A. Scheynius, C. Braun-Fahrlander, M. Waser, and B. Brunekreef. 2005. Bacterial and fungal agents in house dust and wheeze in children: the PARSIFAL study. *Clin. Exp. Allergy* **35**:1272–1278.
- Selim, M. I., A. M. Juchems, and W. Popendorf. 1998. Assessing airborne aflatoxin B1 during on-farm grain handling activities. *Am. Ind. Hyg. Assoc. J.* **59**:252–256.
- Skaug, M. A., W. Eduard, and F. C. Stormer. 2001. Ochratoxin A in airborne dust and fungal conidia. *Mycopathologia* **151**:93–98.
- Sudre, B., M. Vacheyrou, C. Braun-Fahrlander, A. C. Normand, M. Waser, G. Reboux, P. Ruffaldi, E. von Mutius, and R. Piarroux. 2009. High levels of grass pollen inside European dairy farms: a role for the allergy-protective effects of environment? *Allergy* **64**:1068–1073.
- Von Ehrenstein, O. S., E. Von Mutius, S. Illi, L. Baumann, O. Bohm, and R. von Kries. 2000. Reduced risk of hay fever and asthma among children of farmers. *Clin. Exp. Allergy* **30**:187–193.
- von Mutius, E. 2007. Asthma and allergies in rural areas of Europe. *Proc. Am. Thorac. Soc.* **4**:212–216.
- von Mutius, E., C. Braun-Fahrlander, R. Schierl, J. Riedler, S. Ehlermann, S. Maisch, M. Waser, and D. Nowak. 2000. Exposure to endotoxin or other bacterial components might protect against the development of atopy. *Clin. Exp. Allergy* **30**:1230–1234.
- von Mutius, E., and S. Schmid. 2006. The PASTURE project: EU support for the improvement of knowledge about risk factors and preventive factors for atopy in Europe. *Allergy* **61**:407–413.
- Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* **173**:697–703.